2000

The influence of Leptin on metabolic expenditure and thermogenesis during thyroid hormone (T_3) suppression in the obese (OB/OB) mouse

Brian Kimball Underhill

THE INFLUENCE OF LEPTIN ON METABOLIC EXPENDITURE AND THERMOGENESIS DURING THYROID HORMONE (T_3) SUPPRESSION IN THE OBESE (*OB/OB*) MOUSE

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

---

by

Brian Kimball Underhill

June 2000

THE INFLUENCE OF LEPTIN ON METABOLIC EXPENDITURE AND THERMOGENESIS DURING THYROID HORMONE (T_3) SUPPRESSION IN THE OBESE (*OB/OB*) MOUSE

A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

by

Brian K. Underhill

June 2000

Approved by:

[redacted]

Dr. Richard Fehn, Chair, Biology — 6/14/00 Date

[redacted]

Dr. Colleen Talbot

[redacted]

Dr. Gerald Thrush

ABSTRACT

The obese (*ob/ob*) mouse exhibits an inability to synthesize leptin and thus serves as an excellent model to study the effects of leptin through its reintroduction. Leptin is a hormone produced by adipocytes and has been shown to upregulate metabolic expenditure and thermogenesis, induce lipolysis, and cause appetite suppression. It has not been established if leptin is able to exert its actions directly or by stimulating the release of thyroid hormone (T_3). In this study, twenty C57BL/6J-*ob* normal (+/?) and homozygous obese (*ob/ob*) mice between the ages of 8-10 weeks were treated for nine days with either saline vehicle, leptin, iopanoic acid, or leptin plus iopanoic acid (n=5/group). Iopanoic acid was used to prevent leptin from inducing the conversion of T_4 into T_3. Daily measurements of body weight, food consumption, oxygen consumption, carbon dioxide production, and infrared emission were collected in both +/? and *ob/ob* treatment groups. Iopanoic acid was ineffective at reducing serum T_3 in +/? mice, however it successfully reduced T_3 synthesis in *ob/ob* mice relative to vehicle treated *ob/ob* mice.

Vehicle treated *ob/ob* mice displayed a greater food consumption than vehicle treated +/? mice, while both

leptin and iopanoic acid + leptin treatment effectively reduced appetite in *ob/ob* mice below *ob/ob* vehicle treatment groups. With respect to vehicle treated *ob/ob* mice, a lower day 10 body weight was observed in both leptin and iopanoic acid + leptin treated *ob/ob* mice, while the mean body weights for iopanoic acid treated *ob/ob* mice remained unchanged. Iopanoic acid was effective at depressing serum T_3 in *ob/ob* mice which also showed a reduction in body weight similar to that of leptin treated *ob/ob* mice, thus the effect of leptin on weight reduction cannot be attributed to mediation by T_3.

Vehicle treated *ob/ob* mice displayed a lower day 10 radiant body heat with respect to vehicle treated +/? mice (31.5 ± 0.3 °C vs. 33.9 ± 0.4 °C, respectively). Iopanoic acid treated *ob/ob* mice displayed radiant body heat emission similar to those of vehicle treated *ob/ob* mice. However, both leptin and iopanoic acid + leptin treated *ob/ob* mice showed higher radiant body heat than both vehicle and iopanoic acid *ob/ob* treatment groups. This suggests that the thermogenic effects of leptin are similar in both leptin and iopanoic acid + leptin *ob/ob* treatment groups. Iopanoic acid was ineffective at reducing serum T_3 in +/? Mice, however it successfully

reduced T_3 synthesis in *ob/ob* mice relative to vehicle treated ob/ob mice. Thus the reduction of serum T_3 in the iopanoic acid + leptin treatment group shows that this thermogenic influence of leptin occurs without increasing T_3 concentrations.

Vehicle treated *ob/ob* mice had the highest serum T_3 concentrations relative to all other treatment groups, and they also displayed total oxygen consumption rates similar to vehicle treated +/? mice (3.8 ± 0.8 ml $O_2 \cdot min^{-1}$ vs. 3.9 ± 0.6 ml $O_2 \cdot min^{-1}$, respectively). The reduction of T_3 in iopanoic acid treated *ob/ob* mice caused a decline in oxygen consumption relative to vehicle treated *ob/ob* mice ($2.6 + 0.8$ ml $O_2 \cdot min^{-1}$ vs. 3.9 ± 0.6 ml $O_2 \cdot min^{-1}$, respectively). An elevated metabolic rate in both leptin and iopanoic acid + leptin *ob/ob* mice relative to iopanoic acid treated *ob/ob* mice suggests that leptin is acting through T_3 to induce metabolism. Leptin deficient iopanoic acid treated *ob/ob* mice display low serum T_3 relative to vehicle treated *ob/ob* mice, and consequently have low metabolic rates relative to the same control group. Administration of leptin to iopanoic acid + leptin *ob/ob* mice resulted in a similarly elevated metabolic rate without an increasing serum T_3. This confirms that metabolic expenditure can be induced by

leptin and without indirectly increasing serum T_3 concentrations.

ACKNOWLEDGMENTS

Throughout the duration of this project I sought the advice and guidance of many people of whom deserve far more than what I am able to write in the text that follows. Although I am unable to individually identify each person that helped with this project, I sincerely extend my gratitude to all who have encouraged and inspired me to remain focused throughout this program. I especially thank Dr. Richard Fehn, my major advisor, for his guidance and mentoring as a scientist. There is far more to gain from a graduate program than this piece of literature and Dr. Fehn encouraged me to expand upon my skills and explore the numerous opportunities provided to me as a graduate student.

It is most difficult to find the words that describe how much my family has meant to me throughout this thesis. I truly thank my mother, father, and sister for their continued support from the beginning of my education. Most of all I value the strength of our family and the reassurance of knowing that they have been and always will be their for whatever I may need. My family will probably never really be able to truly understand how much they have influenced me during this work, and thus the words "thank

you" do not come close in describing how grateful I am for all they have done. I love them for always being by my side and will always treasure their guidance.

To one of my closest friends during both undergraduate and graduate education, Phil (Homer) Galicia has made so much of this journey truly enjoyable. I look back upon the late nights of studying in my room and staring down the hall for the slightest notion that Phil was ready to take a break - "Homer, some day we'll write a book about it". To our adventures along the Pacific Coast, to the Mexican Sock Tea, and to the stories of ranches in Spain and being chased on horses by bandits to our awaiting ship... I'm happy that we're pals.

I especially thank Dr. Colleen Talbot for her time and patience with much of the technical analysis of the thesis and for serving on my committee. I also thank Dr. Gerald Thrush for serving on my thesis committee and for his assistance on weekends and evenings when my personal work schedule prevented me from contacting him during office hours. I thank both committee members for their enormous work in editing this thesis and for working way beyond an eight-hour day in an effort to assist me in making deadlines.

I thank Carol Smith for always watching over our shoulders and for providing assistance far beyond what could ever have been expected. In addition, I also thank Dwight Gallo and Mike Mahoney for their technical assistance during this project and for being there to help during the duration of both my graduate and undergraduate education.

Now that the last piece of this puzzle has been fit into place, Phil and I must attend to some long awaited beer and reminisce as we keep the glowing flames of a small bon fire lit with memories past.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE: INTRODUCTION

Thermogenesis from Oxidative Metabolism

The development of obesity is a major complication associated with the pathology of Non Insulin Dependent Diabetes Mellitus (NIDDM). Increased body weight is inversely proportional to insulin sensitivity and thus contributes to a decreased ability to transport glucose into the cells resulting in hyperglycemia. Weight loss improves both insulin sensitivity and glycemic control in obese individuals suffering from NIDDM. Thus researchers are looking at mechanisms of inducing metabolic expenditure in order to mobilize stored fat reserves and to ultimately reduce body weight and lead to an improvement of the diabetic state.

The production of heat resulting from overall energy expenditure is categorized by Himms-Hagen (1989) as either obligatory or facultative thermogenesis. The amount of heat produced through obligatory thermogenesis is relatively low and results from thermic effects of food ingestion and energy expended during growth, pregnancy, and lactation. Facultative thermogenesis results from metabolic processes which can be rapidly regulated and includes exercise-stimulated thermogenesis, involuntary

skeletal muscle shivering, and nonshivering thermogenesis. The overall metabolism of all tissues is responsible for maintaining obligatory thermogenesis while facultative thermogenesis is primarily observed in skeletal muscle and brown adipose tissue. Targeting facultative thermogenic tissue to increase energy expenditure has recently become a candidate for the treatment of obesity.

The specific metabolic events contributing to facultative thermogenesis occur as a by-product of oxidative metabolism. A comparison of the change in enthalpy for cellular substrate oxidation to that of ATP hydrolysis shows that the cellular production of heat mainly occurs in the process of ATP production as opposed to ATP usage (Prusiner and Poe, 1968). The donation of electrons to the mitochondrial electron transport system through the oxidation of reduced nucleotides produces a H^+ electrochemical gradient which is then dissipated through the inner membrane bound F_1/F_o/ATP synthase and results in the phosphorylation of ADP. Much research has focused attention on the mechanism and regulation of uncoupling metabolic oxidation from the phosphorylation of ADP.

In order to uncouple oxidative phosphorylation the mitochondrial H^+ electrochemical gradient is dissipated

through an inner membrane protein called uncoupling protein (UCP) (Klingenberg et. Al., 1985). As a result of mitochondrial uncoupling, substrate utilization and oxygen consumption are maintained or can be potentially upregulated while ATP synthesis is no longer tightly coupled to the oxidation of substrates. Scientists have directed their attention towards this inner mitochondrial membrane bound uncoupling protein as a candidate for inducing metabolic expenditure and thus ultimately reducing body weight. The normal role of this protein is to dissipate the hydrogen ion electrochemical gradient established through oxidative metabolism and ultimately result in heat production for the maintenance of body temperature in homeothermic animals (the detailed mechanism will be discussed in the next section). Clinical induction of uncoupling protein (UCP) will permit increased metabolic expenditure while not increasing ATP synthesis, and heat will be produced as a metabolic by-product thus allowing adipose reserves to be increasingly mobilized thus serving as a substrate for this thermogenic process.

Thermogenic Tissues

Facultative thermogenesis is divided into heat produced through muscular activity and excercise,

shivering, and non-shivering thermogenesis (Schmidt-Nelson, 1990). Shivering thermogenesis results from acute involuntary skeletal muscle contraction and aids in maintaining body temperature during drastic environmental temperature changes. Non-shivering thermogenesis was initially described to function in the thermal balance of mammals undergoing cold acclimation, in small mammals arising from hibernation, and in newly born mammals. Brown adipose tissue was initially established as the organ responsible for the mechanism underlying this thermal balance (Himms-Hagen, 1990).

The role of brown adipose tissue as a contributor to whole body thermogenesis is related to its structural composition. Brown adipose tissue consists of approximately 40% brown adipocytes with the remaining mast cells, interstitial cells and preadipocytes that differentiate into brown adipocytes. The characteristic brown color of BAT is the result of high densities of mitochondria with iron containing cytochromes. The rich vasculature of BAT permits the flow of blood from this tissue to be rapidly dispersed to tissues and organs of the body (Himms-Hagen, 1990).

The contribution of BAT to nonshivering thermogenesis in newborn mammals was partly established through studies performed by Heim and Hull (1968) which demonstrated that BAT in newborn rabbits accounts for at least 70% of the increased oxygen consumption following norepinephrine infusion. Himms-Hagen (1989) found a tight correlation between the species-specific thermal requirement and brown fat development. For example, mammals born well developed and able to live independently very early in life, such as the dog and guinea pig, have BAT which is fully developed at birth and atrophies shortly thereafter. However mammals such as the Syrian hamster, which is born poikilothermic, initiates BAT development at approximately 2 weeks of age, when it is able to maintain a constant body temperature. After it was established that BAT is a major contributor to thermoregulation, researchers began proposing it as a potential target to induce metabolic expenditure (Himms-Hagen, 1989). Unfortunately, adult human's lack sufficient quantities of BAT to influence total body temperature and thus specific inductive targeting of BAT could not sufficiently mobilize stored fat reserves and cause a decrease in body weight. Brown adipose tissue is not the only tissue responsible for maintaining heat balance

through non-shivering thermogenesis. Recent research has demonstrated that the uncoupling mechanism responsible for heat production in brown adipose tissue is also found within other thermogenic tissues of the body, namely skeletal muscle (Boss et al., 1997).

When the mechanism of thermogenic uncoupling was discovered to be homologous in skeletal muscle to that of brown adipose tissue researchers began looking at muscle as a target for metabolic induction in humans suffering from obesity. Skeletal muscle constitutes 42% of body mass in both humans and rats and, due to its high mass, greatly contributes towards whole body oxygen consumption relative to other tissue types. Therefore many investigators support it as a primary tissue for clinically targeting the induction of metabolic uncoupling and the treatment of obesity.

History of Uncoupling Protein

During the early to mid 1970's research focussed on the influence of fatty acids on the induction of oxidative uncoupling and nucleotides inducing recoupling in BAT mitochondria (Cannon et al., 1973; Rafael et al., 1969; Hittelman et al., 1969). Heaton et al. (1978) identified a 32,000-M_r protein responsible for proton conductance.

However it remained unsettled as to whether this newly discovered protein was responsible for the nonshivering thermogenesis attributed to brown fat. Support for this came from an observed correlation between the thermogenic output and the newly discovered 32,000-M_r protein in animals which vary in thermogenic capacity (Nicholls and Locke, 1984). For instance, the decline in nonshivering thermogenesis during guinea pig development correlates with the decline in both the amount of the 32,000-M_r protein, and the proton conductance of the inner mitochondrial membrane (Nicholls and Locke, 1984). This particular uncoupling protein was isolated by Lin and Klingenberg (1982) and found to be solely expressed in brown adipose tissue (BAT).

Several observations led to the hypothesis that mitochondrial uncoupling of oxidative phosphorylation exists in tissues other than BAT. It was observed that the inner mitochondrial membranes of isolated intact liver cells were permeable to protons and that cellular oxygen consumption, for the purpose of maintaining the electrochemical gradient against the proton leak, accounted for approximately 26% of the total resting respiration rate in isolated liver cells and approximately 52% of resting rat skeletal muscle (Brand et al., 1994; Rolfe and Brand,

1996). These studies confirmed that the proton leak in rat liver, skeletal muscle and heart contribute to an average 20% of the standard metabolic rate in the adult rat (Nobes et al., 1990; Brown et al., 1990; Brand et al., 1994; Rolfe & Brand, 1996). The proton leak in these tissues, as well as in brain and kidney, suggested the presence of a mechanism of uncoupling in tissues other than that previously described in BAT (Rolfe et al., 1994). Further evidence supporting the need for a mechanism to regulate energy expenditure in tissues other than BAT was that adult humans have very little BAT (Cunningham et al., 1985; Garruti et al., 1993; Lean et al., 1986). Furthermore, although obese mice demonstrate atrophied BAT and low thermogenic output, mice where UCP is not expressed in BAT are not always obese. This compounding evidence led to studies in which several other UCP's, with high homology to the UCP expressed in BAT (now defined as UCP1), were identified and characterized (table1): UCP2 (Fleury et al., 1997; Gimeno et al., 1997; Boss et al., 1997), UCP3 (Boss et al., 1997; Solanes et al., 1997), and UCP4 (Mao et al., 1999).

Table 1. Uncoupling proteins and the tissue types where they have been isolated. BAT=brown adipose tissue, WAT=white adipose tissue, SM=skeletal muscle

Uncoupling Protein Type	**Tissue**
UCP2	BAT, WAT, SM, Heart Kidney, Lung, Placenta Lymphocytes
UCP3	BAT, SM
UCP4	Brain

Characteristics of Uncoupling Protein

All UCP's belong to the gene family of mitochondrial anion carrier proteins which include the ATP/ADP translocator, the phosphate carrier, the 2-oxygluturate /malate carrier, and the citrate carrier (Palmier, 1994). All members of this family have three highly conserved repeated domains. Two transmembrane domains exist within each repeating unit and a highly conserved basic motif, containing the amino acid residues allbnb (a=acidic, l=lipophilic, b=basic, n=neutral), occurs

after the first transmembrane domain of each repeat (Klingennberg, 1993). Human UCP1 and UCP2 are 59% identical at the amino acid level, and UCP3 is 57% and 71% identical to UCP1 and UCP2, respectively (Vidal-Puig et al., 1997). UCP3 exists in a both short and a long forms (Boss et al., 1997; Solanes et al., 1997); the short form of UCP3 is missing 37 amino acids at the C-terminus that are present in the long form and which are also found in UCP1 and UCP2. There is no change in the ratio of long to short form of the transcript in obese and lean subjects before and after caloric restriction (hypocaloric diet of 1045kJ/day for 5 days) suggesting the absence of alteration of gene regulation and alternative splicing in the skeletal muscle of obese subjects (Millet et al., 1998). Separate studies by Boss et al. (1997) and Solanes et al. (1997) suggest the C-terminus present in the long form, but not in the short form, contains a transmembrane domain that may function to correctly insert UCP into the membrane.

The mechanism by which uncoupling protein facilitates the dissipation of the H^+ gradient across the inner mitochondrial membrane has been described to be driven via fatty acid cycling (Garlid et al., 1996; Jezek et al., 1998). The proposed mechanism of fatty acid cycling works

through a membrane bound domain of the UCP that contains an anion conductance pathway. The transportating of H^+ begins with protination of a membrane bound fatty acid which then flips to the matrix and delivers a H^+ down its gradient via nonionic diffusion. The bilayer energy barrier is too high to allow the flux of the Fatty acid (FA) anion back across the membrane. The energy barrier is reduced by the anion conductance pathway of the UCP. The FA anion flip-flops to the other side of the membrane, along the provided conductance pathway, and becomes protinated for a nonionic diffusion to complete the cycle. UCP2 and UCP3 are suggested to both facilitate the transport of H^+ through the same mechanism of fatty acid cycling (Jezek et al., 1998).

Leptin as a Satiety Factor and Regulator of Metabolic Expenditure

The *ob* gene encodes a 167 amino acid peptide called leptin which is expressed in brown and white adipose tissue, placental tissue, and regions along the gastrointestinal tract (Prolo et al., 1998; Auwerx and Staels, 1998). Leptin reduces appetite and induces metabolic expenditure by regulating the level of central nervous system activity, specifically the expression of hypothalamic neuropeptide Y (NPY). The NPY is a stimulator

of feeding and inhibitor of brown fat uncoupling protein thermogenesis activity (Kotz et al., 1998). Leptin has been shown to inhibit NPY release from the hypothalamic arcuate nucleus and subsequently results in decreased feeding and an increased BAT UCP1 expression (Kotz et al., 1998).

Leptin's action on peripheral cell activity is the result of both direct leptin receptor binding and activation of the sympathetic nervous system. Catecholamine levels have been shown to rise in a dose dependent manner with leptin administration. Adipocyte beta-adrenergic receptor binding of catecholamines activates intracellular adenylate cyclase and, through a signal transduction cascade, activates triglyceride lipase catalyzing the breakdown of triacylglycerol. While sympathetic nerve activity increases adipocyte lipolytic activity, simultaneous adiopocyte leptin gene expression is inhibited. (Hua et al., 1997). Leptin acts similarly in other peripheral tissues to cause the depletion of cellular triglycerides. Shimabukuro et al. (1997) demonstrated that leptin administration causes skeletal muscle, liver, and pancreas cells to break down intracellular triglycerides. Furthermore, simultaneous upregulation of free fatty acid

(FFA) oxidation with leptin infusion was suggested by the absence of an increase in serum FFA content, that should have resulted from the increased cellular lipolysis. Leptin induced depletion of FFA content is supported through a simultaneous upregulation of ß-oxidation (Shimabukure et al., 1997). An interesting study performed by Wang et al. (1999) demonstrates through adipocyte cell culture that leptin and norepinephrine independently induce lipolytic activity. Interestingly, norepinephrine induction of lipolysis results in free fatty acid and glycerol release, while leptin lipolytic induction was followed by only glycerol release thus supporting a leptin induced ß-oxidation. While leptin is inducing fat oxidation, a simultaneous inhibition of acetyl-CoA carboxylase activity, the rate-limiting enzyme of fatty acid synthesis, by leptin subsequently leads to a reduction of malonyl-CoA (Bai et al., 1996). Since malonyl-CoA directly inhibits carnitylacyltransferase I, an enzyme catalyzing the translocation of fatty acid into the mitochondrial matrix, oxidation will ultimately increase.

Leptin has also been demonstrated to alter peripheral tissue metabolic expenditure through changes in the magnitude of oxidative uncoupling. Leptin's action as a

regulator of metabolic expenditure on peripheral tissue seems to be partly mediated through sympathetic nervous activity. Haynes et al. (1997) showed that leptin increased sympathetic nerve activity to brown adipose tissue, renal tissue, and the hindlimb. This upregulation of catecholamine production induces lipolytic activity thus resulting in increased serum fatty acid levels (Wang et al., 1999). Hwang and Lane (1999) showed that administration of fatty acids to skeletal muscle cells in vitro results in a time and concentration dependent increased UCP3 mRNA expression. Intralipid infusion mimics the rise in plasma free fatty acid levels that occurs as a result of adipocyte fatty acid mobilization during a fasted state. Weigle et al. (1998) elevated serum free fatty acid levels in fed animals through intralipid infusion (20% Intralipid) and observed an increased skeletal muscle UCP3 mRNA. In the same study, fasting rats were observed to increase serum FFA levels 3-fold, accompanied by increased skeletal muscle expression of UCP3 and UCP2 mRNA.

The synthesis of catecholamines occur in the CNS, sympathetic postganglionic neurons, and adrenal chromaffin tissue. While both intravenous and intracerebroventricular injection (ICV) of leptin results in increased plasma

catecholamine (norepinephrine and epinephrine), ICV injection requires approximately 100 fold less leptin to increase catecholamine secretion (Satoh et al., 1999). Other ICV leptin injection studies support the idea that fats were mobilized to fuel an increased uncoupling through an observed reduction in the respiratory quotient, shifting from carbohydrate to fat metabolism (Ghibaudi et al., 1996). During the ICV leptin injection study performed by Satoh et al. (1999), both norepinephrine and epinephrine plasma levels remained elevated and without change for 3 hours post injection. It is also shown that a continuous leptin ICV infusion of 4 days (12 μl/day) is able to maintain high levels of UCP1, UCP2, and UCP3 mRNA.

Leptin's Action Through Thyroid Hormone

Thyroid hormone is synthesized in the thyroid gland through the coupling of iodinated tyrosines to yield both thyroxine (3,4,3',5'-Tetraiodothyronine, T_4) and, to a lesser amount, triiodothyronine (3,5,3'-Triiodothyronine, T_3). Both T_4 and T_3 are secreted into the circulation and while T_3 is the physiologically active form, T_4 must be converted to T_3 in order to elict most responses. The conversion of T_4 to T_3 is catalyzed in the peripheral tissues by either type I or type II T_4 5'-monodeiodinase

(5'-D). Type I 5'-D is responsible for most of the conversion of T_4 and is found in the kidneys and liver, whereas type II is found in BAT, brain, and pituitary gland.

T_3 stimulates mitochondrial metabolism and oxygen consumption and thus increases the level of cellular ATP. T_3 also increases the expression of plasma membrane bound Na^+/K^+-ATPase expression. Thus both increased levels of ATP along with T_3 stimulated Na^+/K^+-ATPase expression provides the basis for the sodium pump theory of thermogenesis, which describes the process of ATP hydrolysis by the Na^+ pump as an exothermic reaction yielding the production of heat for the maintenance of body temperature. Thyroid hormone (T_3) has also been shown to elicit a thermogenic effect through increased mitochondrial proton leak in isolated liver cells (Rolfe and Brand, 1997). Furthermore T_3 induced expression of UCP1, UCP2, and UCP3 mRNA is suggested to contribute to thermogenesis (Boss et al., 1997; Gong et al., (1997).

Fehn et al. (2000) reported a similar degree of metabolic stimulation by both leptin and T_3 in the *ob/ob* mouse. However, the thermogenic and metabolic actions of leptin through T_3 have never been described. Our laboratory

has demonstrated, through histological analysis of thyroid glands in obese (*ob/ob*) mice, an enhanced follicular cell activity and enlarged colloids with leptin treatment, suggesting increased thyroid hormone in response to leptin (Fehn et al., 1999). In addition serum it has demonstrated increased serum leptin levels in response to T_3 treatment. Thus since both leptin and T_3 independently induce thermogenic uncoupling and T_3 synthesis seems to be dependent upon serum leptin levels, it is possible to predict that the thermogenic effect of leptin is through the induction of T_3 synthesis.

Skeletal Muscle Metabolic Uncoupling

Brown adipose tissue (BAT) is well documented to be a major site of nonshivering thermogenesis in rodents however its control is defective in most animal models of obesity. Himms-Hagen (1990) reports that BAT of obese rodents is thermogenically inactive and exhibits a characteristically depressed ability to synthesize uncoupling protein, thus the requirement to thermoregulate in obese animals implies the existance of uncoupling protein in other tissues. Furthermore, the presence of relatively small amounts of BAT in humans supports the need for a mechanism of nonshivering thermogenesis in tissues other than BAT. The

discovery of UCP3, expressed primarily in skeletal muscle, led investigators to target it as a potential means of clinically regulating metabolic expenditures to treat obesity. Interestingly, both leptin and T_3 similarly induce the genetic expression of skeletal muscle UCP3 (Liu et al., 1998; Gong et al., 1997).

Human skeletal muscle lipolysis is induced through $ß_2$-adrenoceptor agonist and not $ß_1$- or $ß_3$- specific agonists (Hagstrom-Toft et al., 1998). It has not been clearly established if T_3 permissively induces the expression or activity of skeletal muscle $ß_2$-adrenergic receptor in order to permit a catecholamine stimulated skeletal muscle thermogenic response. Neither has it been established if leptin is able to directly alter skeletal muscle uncoupling in the absence of centrally mediated adrenergic influence. Thus it would be of interest to experimentally prevent the up-regulation of thyroid hormone by leptin and thereby remove the permissive action of T_3 on peripheral tissue adrenergic receptors. This would allow investigators to evaluate leptin's action on peripheral tissue activity through adrenergic activity and, in the absence of thyroid hormone's additional influence, would also yield a better

understanding of the direct action of leptin on peripheral tissue uncoupling.

Experimental Design

The C57BL/6J obese (*ob/ob*) mouse exhibits an inability to synthesize functional leptin and is reported to display hypothyroidism (low T_3, high T_4) from 3 to 6 weeks of age (Oh and Kaplan, 1994). These mice also exibit a reduced oxygen consumption and low body temperature (Kaplan and Leveille, 1974). Daily injection of *ob/ob* mice with leptin reduces body weight and food intake, while increasing metabolic rate (Pelleymounter et al., 1995). Studies from our laboratory have shown that with leptin administration the *ob/ob* mouse increases oxygen consumption rates and thermogenesis (Fehn, et al., 1999). The mechanism of leptin stimulation in peripheral tissue metabolic and thermogenic activity in the absence of T_3 has not been determined and is the focus of this particular research.

As discussed earlier, T_3 is synthesized through the deiodination of T_4 by $T_4$5'-monodeiodinase. In order to evaluate the ability of leptin to stimulate metabolism and thermogenesis we needed to block leptin's ability to stimulate T_3 synthesis. Iopanoic acid was used to block renal and hepatic $T_4$5'-monodeiodinase. Obese *ob/ob* and

normal +/? mice were used to measure oxygen consumption, carbon dioxide production, radiant body heat, and food consumption while given either saline vehicle, leptin, iopanoic acid, or leptin in addition to iopanoic acid.

CHAPTER TWO: MATERIALS AND METHODS

Animals

Twenty of the C57BL/6J-*ob* normal (+/?) and homozygous obese (*ob/ob*) female mice were ordered from Jackson Laboratories (Bar Harbor, Maine). All mice were ordered to arrive at 8-10 weeks of age received initial treatment three days after arrival. Mice were housed at five animals per cage of the same genotype and treatment group and maintained at approximately 23 °C under a 14:10 light:dark cycle. Water and Teklab 4% rodent chow were available *ad libitum*.

Experimental Design

Recombinant mouse leptin was purchased from R&D Systems (#498-OB). The leptin formulation was lyophilized from a filtered solution in phosphate buffered saline (PBS). Leptin was reconstituted by adding 15mM sterile HCl (2.5 ml/5mg leptin) to a vial and allowing the protein to dissolve. Once leptin was completely dissolved, 7.5 mM of sterile NaOH (1.5 ml/5mg vial) was added to bring the pH to approximately 5.2. The resulting suspension was aliquoted into microcentrifuge tubes and stored at -20 °C until used. Iopanoic acid was purchased from SIGMA Biochemicals (Product Number I 2013) and was prepared immediately before

each use by adding 6.25 ml of 0.1M NaOH to 125 mg of the drug. Once the drug was dissolved, it was titrated with 0.1M HCl to approximately pH 7.8 and then diluted with distilled water to a volume of 12.5 ml and protected from light.

Mice were organized into 4 groups with 5 animals per group. Each group received daily intraperitoneal injections of one of the following: leptin (3 μg/g body weight; 2.4 μl/g body weight), iopanoic acid (50.0 μg/g body weight; 5.0 μl/g body weight), or both leptin and iopanoic acid as a double injection (as described above). Control animals received intraperitoneal injections of leptin vehicle (PBS pH 5.2; 2.4 μl/g body weight). Injections were administered between the hours of 0800 hours and 1200 on nine consecutive days (day 1 through day 9) following readings of O_2 consumption, CO_2 production, radiant body heat, and body weight determinations.

Percent change in body weights with respect to day 1 were determined for each animal and statistical analysis (ANOVA) was performed on last day percent change. In order to statistically evaluate all values of percent change as positive numbers, a constant of 0.25 was added to the

percent change of each animal and then arcsin transformation was applied to the values (Zar, 1984).

Metabolic Rate Determination

Oxygen consumption and carbon dioxide production were measured using open flow respirometry with an Ametek S-3A/II O_2 analyzer and an Ametek CD-3A CO_2 analyzer, respectively. Room air was utilized as a reference gas and assumed to contain 20.96% O_2 and 0.03% CO_2. Readings were taken at a constant flow rate of 400 ml per minute. Mice were placed in an open flow container and the fractional gas content was recorded at the end of 2 minutes, a time which was determined to provide stable readings. Animals were weighed after metabolic determination. Results are reported as total oxygen consumption (ml $O_2 \cdot min^{-1}$). Respiratory quotients (RQ, ml CO_2/ml O_2) were determined from oxygen consumption and carbon dioxide production in order to evaluate metabolic substrate usage.

Food Consumption

Daily food consumption was determined for each treatment group by weighing any uneaten food in both the tray and inside mouse cage (moisture accumulation was not detected in uneaten food). Food was weighed under the assumption that moisture from saliva and urnine negligably

altered weight. Food consumption measurements were begun the same day of treatments and thus pretreatment data are not available. Mean daily food consumption (grams/day) was measured for each group of five mice on each day of the study. Data was analyzed for the last three days (days 8-10).

Radiant Body heat Determination

Radiant body heat was determined daily using a thermocouple thermometer (Dual J-T-E-K, model 91100-40) with an infrared probe (Cole Parmer, model 39652-10 K). The probe was positioned approximately 2 cm from the ventral surface of the animal's lower abdomen and held until a constant value was obtained (approximately 15 seconds). Radiant body heat was recorded in °C and used as an indicator of thermogenesis.

Blood Collection and Serum Thyroid Hormone Determination

Blood was collected via cardiac puncture, and then sacraficed by CO_2 euthanasia. Blood samples were transferred to 1.5 ml microcentrifuge tubes and placed on ice until clotted. Serum was separated from the clot by centrifugation (15,000 xg) and stored at -20 °C until assayed. All samples were subjected to radioimmunoassay quantification of T_3 and T_4 using the ^{125}I T_3 Solid Phase

Component System and the ^{125}I T_4 Mab Solid Phase Component System (ICN Pharmaceuticals, T_3 06B-254215; T_4 06B-254011). Standards for the T_3 kit ranged from 0 ng/dL to 800 ng/dL L-Triiodothyronine in human serum with sodium azide. Standards for the T_4 kit ranged from 0 μg/dL to 20 μg/dL L-thyroxine in human serum with sodium azide.

Statistical Analyses

All day 10 results were subjected to multifactorial analysis of variance (ANOVA) and Duncan's Multiple Range Test (Mason et al., 1989). Statistical significance is shown in a chart below each figure or table where treatment groups intersect (S = significance; NS = not significant). Missing data points occurred in the following thyroid hormone groups: vehicle treated +/? T_3, and iopanoic acid treated *ob/ob* T_3. Subsequently, these same groups had missing data for the T_3/T_4 determinations. After calculating the ratio (T_3/T_4) for vehicle treated *ob/ob* mice, there was an outlier that was also removed as a missing data point. Missing data points were estimated by the method of Zar, (1984).

$$\hat{x}_{ij} = \frac{aA_i + bB_j - \sum_{i=1}^{a}\sum_{j=1}^{b}\sum_{k=1}^{n} x_{ijk}}{N + 1 - a - b},$$

For each group where missing data occurred, ANOVA was calculated as n=4. All other ANOVA calculations were reported using n=5 (mean $\pm$ SD). P values 0.05 are declared significant.

RESULTS

Serum Thyroid Hormones

Serum thyroid hormone (T_3) concentrations in vehicle treated 8-10 week old *ob/ob* mice were greater than in the normal vehicle treated group (table 2). Obese mice treated with leptin, iopanoic acid, and iopanoic acid + leptin displayed a lower serum T_3 concentrations relative to vehicle treated *ob/ob* mice and were not different from nomal treatment groups. Iopanoic acid + leptin treated *ob/ob* mice displayed a higher serum T_4 than leptin treated +/? mice. The ratio of T_3/T_4 was also evaluated as an indication of the T_4 converted to T_3 through the action of T_4 5'-monodeiodinase. No difference was observed in the T_3/T_4 ratio for any of the treatment groups.

Daily Food Consumption

Daily food consumption was measured to confirm leptin's role as a satiety factor and these data are shown in figure 1. Vehicle treated *ob/ob* mice have a higher food consumption than +/? vehicle treated mice. Leptin treatment resulted in a depressed food consumption in *ob/ob* mice relative to vehicle treated *ob/ob* mice. Food consumption by iopanoic acid treated *ob/ob* mice remained unchanged with respect to vehicle treated *ob/ob* mice,

however it was greater than leptin treated *ob/ob* mice. Iopanoic acid + leptin treated *ob/ob* mice displayed a similar food consumption to that of leptin treated *ob/ob* mice, and it was significantly lower than both vehicle and iopanoic acid treated *ob/ob* mice.

Body Weight Analysis

Day 10 body weights for normal (+/?) and obese (*ob/ob*) mice are shown in Figure 2. All normal (+/?) treatment groups display equivalent body weights while vehicle treated *ob/ob* mice had higher body weights than vehicle treated +/? mice. Leptin treatment in *ob/ob* mice resulted in lower body weights with respect to vehicle treated *ob/ob* mice. Obese mice treated with iopanoic acid had body weights equal to vehicle treated *ob/ob* mice. Administration of iopanoic acid with leptin to *ob/ob* mice caused the lowest body weights with respect to all *ob/ob* treatment groups.

Percent change in body weight relative to day 1 is used to show the relative changes in body weights for each treatment group (figure 3 a and b). Statistical analysis was performed on day 10 percent changes by using arcsin transformation. Percent change in body weights for vehicle, leptin, iopanoic acid, and iopanoic acid + leptin

treated normal (+/?) mice displayed a similar slight decrease, while iopanoic acid + leptin decreased more than all other treatment groups by day 10 (figure 3a). Relative to vehicle treated *ob/ob* mice, all *ob/ob* treatment groups displayed a lower absolute body weight. Iopanoic acid treated *ob/ob* mice show a smaller percent change in body weight relative to *ob/ob* vehicle, while leptin and iopanoic acid + leptin display lower body weights . Vehicle and iopanoic acid treated *ob/ob* mice gained weight over the course of the study, while leptin and iopanoic acid + leptin treated *ob/ob* mice lost weight.

Thermogenic Response

Figure 4 shows that, on day 10, vehicle treated normal +/? mice have a higher radiant body heat than vehicle treated obese *ob/ob* mice. Leptin treated *ob/ob* mice have a higher mean radiant body heat than vehicle treated *ob/ob* mice, however the difference was not significant. Iopanoic acid treatment did not result in a change in radiant body heat in *ob/ob* mice relative to vehicle treated *ob/ob* mice, however the administration of iopanoic acid with leptin resulted in a higher thermogenic response relative to vehicle treated *ob/ob* mice.

Oxygen Consumption Analysis

Oxygen consumption rate (ml $O_2 \cdot min^{-1}$) was measured as an indicator of metabolic expenditure. All day 10 normal (+/?) treatment groups displayed no difference in total oxygen consumption rate (figure 5). Leptin treatment in *ob/ob* mice did not change oxygen consumption rate relative to vehicle treated *ob/ob* mice while iopanoic acid treated *ob/ob* mice had lower oxygen consumption rates relative to both vehicle and leptin treated *ob/ob* mice. Leptin treatment coupled with iopanoic acid prevented the decrease in oxygen consumption rate relative to iopanoic acid treatment alone.

Respiratory Quotient

Respiratory quotients (RQ) were used to assess which substrate (fat, carbohydrate, or protein) was primarily metabolized by mice in each treatment group (figure 6). Day 10 vehicle treated normal mice had RQ values below that which would be calculated for pure fat metabolism. All other normal and obese day 10 treatment groups had RQ values that suggested fat metabolism.

CHAPTER FOUR: DISCUSSION

Previous research has shown that food restricted obese *ob/ob* mice display depressed levels of T_3 and T_4 relative to normal mice (Oh and Kaplan, 1994). Results of the current study contrast with Oh and Kaplan (1994) in that obese (*ob/ob*) mice display significantly higher levels of serum T_3 than vehicle treated normal (+/?) mice, whereas T_4 levels in +/? and *ob/ob* mice are equal. However, food restriction has been shown to reduce plasma T_3 levels (Cokelaere et al., 1996; Oberkotter and Rasmussen, 1992), and since the mice used in this study had free access to food, the differences in serum thyroid levels observed between studies may reflect the effects of food restriction.

Leptin has been shown to induce metabolic expenditure (Pelleymounter et al., 1995) and previous research in the host laboratory suggests that its action on peripheral tissues may potentially be regulated through the induction of thyroid hormone (Fehn et al., 1999). The obese *ob/ob* mouse lacks functional leptin and was initially suspected to display reduced serum T_3 levels with respect to normal mice, thus making it an excellent model to study the effects of leptin replacement on T_3 production. Iopanoic acid was used to inhibit T_4 5'- monodeiodinase and thus

prevent leptin's proposed ability to increase the production of T_3. All normal (+/?) mice displayed equal quantities of serum T_3 regardless of treatment, in spite of the fact that +/IOP treated mice were predicted to display significantly depressed T_3 concentrations. Interestingly, iopanoic acid was effective at reducing serum T_3 levels in *ob/ob* mice relative to vehicle treated *ob/ob* mice. The form of iopanoic acid used in this study was different than previously used in our laboratory and thus the pH stability and effective dosages may have been different. Ideally a preliminary iopanoic acid dose response analysis should have been performed.

In the current study leptin treated *ob/ob* mice displayed a lower serum T_3 concentrations as compared to vehicle treated *ob/ob* controls (table 2). These results and those reported by Fehn et al. (1999) suggest that while the thyroid gland activity is increased in leptin treated *ob/ob* mice, the conversion of T_4 to T_3 remains low. It should be noted that Fehn et al. (1999) reported increased thyroid gland activity in leptin treated *ob/ob* mice and did not measure serum thyroid hormone levels. The unexpected hyperthyroid state observed in vehicle treated *ob/ob* mice may be a mechanism to maintain a normal metabolism to

compensate for lack of leptin. The data show that iopanoic acid was effective at lowering serum T_3 in *ob/ob* mice relative to vehicle treated *ob/ob* mice.

Iopanoic acid was administered to inhibit the conversion of T_4 to T_3, through blocking the function of T_4 5'- monodeiodinase and a reduction in the T_3/T_4 ratio with respect to vehicle was expected. Ratios of T_3/T_4 display no statistical difference among either +/? or *ob/ob* treatment groups. However, the negative feedback action of T_4 on pituitary thyrotropin production might have lowered T_4 and produced a normalization of the T_3/T_4 ratio that would have otherwise decreased due to the decline in T_3 (Hadley, 1982).

Normal (+/?) vehicle treated mice represent those whose appetite and metabolic expenditure are uninfluenced by exogenous hormonal manipulation. Halaas et al. (1995) reported that leptin treatment (12.5 μg/g body weight) of normal mice resulted in decreased food intake. They attributed the reduced food intake to a decreased metabolic demand resulting from a leptin induced reduction of body weight. In the current study, leptin treatement (3 μg/g body weight) of +/? mice did not cause a reduction of body weight relative to vehicle treated +/? mice and thus their exists no alteration in metabolic demand, as suggested by

Halaas et al. (1995), and therfore a change in food consumption did not occur relative to vehicle treated controls. Daily food consumption for iopanoic acid and iopanoic acid + leptin treated +/? mice were also unchanged compared to vehicle treated controls.

Leptin's role as a satiety factor is shown in vehicle treated *ob/ob* mice whose inability to produce functional leptin results in greater food consumption with respect to normal mice (figure 1). The administration of leptin inhibits feeding as seen by the observed depression of daily food consumption relative to vehicle treated *ob/ob* mice. Iopanoic acid treated *ob/ob* mice had lower levels of serum T_3 relative to vehicle treated *ob/ob* mice (table 2). The inability of these mice to produce functional leptin as well as an inability to increase synthesis of T_3 most likely accounts for the depressed metabolic rate (figure 5). Since the metabolic rates decreased in iopanoic acid treated *ob/ob* mice while food consumption remained unchanged, it was expected that the iopanoic acid treated *ob/ob* mice would display a body weight above that of vehicle treated *ob/ob* mice. This did not occur and could possibly be due to the chronic diarrhea observed in iopanoic acid treated animals which continued for

approximately 6-7 days from the beginning of treatment. The combined treatment of leptin and iopanoic acid depressed food consumption below that observed in either leptin or iopanoic acid treated *ob/ob* mice.

Day 10 body weights for normal (+/?) mice were unaffected by any treatment (figure 2). All +/? treatment groups consume similar amounts of food (figure 1), thus supporting no observed change in body weights. The percent change in body weight with respect to day 1 was calculated from actual body weights and reported in figure 3 (a,b). Iopanoic acid + leptin treated +/? mice showed a lower percent change in body weight relative to all other +/? treatment groups. Obese (*ob/ob*) mice lack the ability to synthesize functional leptin and this accounts for the inability to effectively suppress appetite, which contributes to the increase in body weight observed over the course of the study. Both leptin and T_3 have been described as inducers of metabolic expenditure (Lombardi et al., 1997; Mistry et al., 1997), but since *ob/ob* mice lack leptin, the high T_3 levels observed in vehicle treated *ob/ob* mice relative to vehicle treated +/? mice is possibly a physiological compensation for lack of leptin and thus accounts for the similar total oxygen consumption in this

group relative to vehicle treated +/? mice (figure 5). Leptin treatment in *ob/ob* mice causes a significant reduction in body weight compared to vehicle treated *ob/ob* mice. This leptin effect is most likely the result of appetite suppression, which is consistent with the observed reduction in food consumption (figure 1), and increased lipolytic activity (Shimabukuro et al 1997). The inability of leptin to elevate radiant body heat and oxygen consumption in leptin treated *ob/ob* mice lends support to the idea that the body weight reduction was not the result of induced metabolic expenditure. Iopanoic acid treated *ob/ob* mice lack leptin and display reduced serum T_3 relative to vehicle treated *ob/ob* mice (table 2). Iopanoic acid treated *ob/ob* show a high body weight relative to leptin and iopanoic acid + leptin *ob/ob* mice due to the combined absence of leptin as a satiety factor and the reduced metabolic rate associated with no leptin and the inability to compensate through increasing thyroid hormone (T_3). Forty hours of fasting has been shown to reduce serum T_4 and T_3 in rats by 28% and 38%, respectively (Kmiec et al., 1998). Interestingly, after a 48 hour fast, both leptin binding to the arcuate nucleus (ARC) and the density of leptin receptors on the ARC has also been shown to increase

by two-fold (Baskin et al., 1999). In the current study iopanoic acid reduced serum T_3 by 50% relative to vehicle treated mice. One might speculate that the expression of leptin receptors on the arcuate nucleus are upregulated by low levels of T_3, and thus increases leptin sensitivity. This would support both a greater inhibition of feeding leading to a larger decrease in body weight in the iopanoic acid + leptin treated *ob/ob* mice relative to *ob/ob* mice treated with iopanoic acid alone.

Oxygen consumption and carbon dioxide production were used to calculate the respiratory quotient (RQ) to provide an indication of the metabolic substrate. An RQ value of 0.71 indicates that fats are being used as the primary source of fuel. Normal +/? vehicle treatment group displayed a day 10 RQ value below 0.71 (0.62 + 0.05). An RQ value below 0.71 is indicative of either metabolic alkalosis associated with hyperventilation, or a large oxygen uptake after exercise so as to replenish an oxygen debt (Schmidt-Nielson, 1990). It is unclear as to whether an RQ value below 0.71 in vehicle treated +/? mice was due to experimental error or the above mentioned variables. All other +/? treatment groups had day 10 respiratory quotients that suggested fats as a primary source of fuel

(figure 6). All day 10 obese (*ob/ob*) mice display RQ values that also indicate the utilization of fats. All RQ data seems unreliable.

Intracerebroventricular and systemic injections of leptin have both been shown to increase metabolic rate (Pelleymounter et al., 1995; Mistry et al., 1997; Ghibaudi et al., 1996). Although the pathway by which leptin causes metabolic induction has not been fully identified, research has provided evidence that both sympathetic activation via the central nervous system (Haynes et al., 1997) and T_3 induction (Fehn et al., 1999) remain the two major possibilities. A reduction in serum T_3 accompanied a depressed oxygen consumption in iopanoic acid treated *ob/ob* mice relative to vehicle treated *ob/ob* mice, suggesting that T_3 was a major determinant of metabolic rate in fed *ob/ob* mice (figure 5). Interestingly, by maintaining a depressed T_3 state, relative to vehicle *ob/ob* mice, and reintroducing leptin in iopanoic acid + leptin treated *ob/ob* mice, we observed a similar oxygen consumption relative to vehicle treated *ob/ob* mice (figure 5). This suggests that the leptin induced metabolic recovery does not require an upregulation of T_3. Thus, sympathetic activation apparently plays a much greater role in leptin

influenced metabolism than does T_3. Furthermore, while T_3 levels remain depressed in both leptin and iopanoic acid + leptin treated *ob/ob* mice relative to vehicle treated *ob/ob* mice, mean radiant body heat is higher in both groups relative to the same control group (it should be noted that leptin elevated radiant body heat relative to vehicle treated *ob/ob* mice was not significant).

Studies support the thermogenic role of leptin acting through increased thermogenic activity and an upregulation of uncoupling protein 3 (Gong et al., 1997; Ghibaudi et al., 1996). Furthermore, it has been clearly established that leptin functions to increase metabolic rate through the upregulation of uncoupling proteins and oxygen consumption in the *ob/ob* mouse (Cinti et al., 1997; Mistry et al., 1997). However, it has not been shown whether leptin is able to act on peripheral tissue upregulation of thermogenic activity or corresponding metabolic rate in the absence of T_3 induction. Leptin induced heat production and oxygen consumption in iopanoic acid + leptin *ob/ob* mice relative to iopanoic acid *ob/ob* mice may be partially attributed to an upregulated proton leak via skeletal muscle uncoupling.

The heat produced by the body is used to maintain a relatively constant body temperature. Thus any excess heat that is produced as a result of either extrinsic factors, such as leptin or thyroid hormone administration, or intrinsic stimulation is emitted from the body surface. Infrared emission was used as an indicator of radiant body heat with the argument that heat emitted from subcutaneous vascularization is uninhibited by alterations in fat insulation. Therefore, radiant body heat was used as an accurate indicator of heat production in excess to that which is used in maintaining a core body temperature. A lower radiant body heat for vehicle treated *ob/ob* mice relative to +/? mice is interpreted as a reduced ability to produce excessive heat to that which is used to maintain constant body temperature and thus results in a lower infrared emission. Interestingly, the high T_3 levels associated with vehicle treated *ob/ob* mice indicates that the low radiant body heat observed in this group, relative to vehicle treated +/? mice, is not due to the T_3 state. Leptin's role as an inducer of metabolic expenditure suggests that an increased radiant body heat should be observed for leptin treated *ob/ob* mice relative to vehicle treated *ob/ob* mice. However, radiant body heat in leptin

treated *ob/ob* mice was not elevated with respect to vehicle treated *ob/ob* mice. In comparison, Pelleymounter et al. (1995) observed an significant increase in core body temperature, however they administered leptin to *ob/ob* mice at over 3 times concentration used in this study and for over 3 times as long. The lack of a detectable thermogenic response in the current study is likely due to the lower dose of leptin used and because the infrared emission method measures excess radiant heat rather than core body temperature.

Leptin treatment alone (3 μg/g) did not change oxygen consumption in *ob/ob* mice relative to vehicle treated *ob/ob* mice. However, Pelleymounter et al.(1995) observed a leptin induced oxygen consumption in *ob/ob* mice at 10 μg/g but not at 0.1 μg/g or 1.0 μg/g. Interestingly, while iopanoic acid treatment reduced oxygen consumption in *ob/ob* mice relative to vehicle treated *ob/ob* mice, simultaneous administration of leptin preserved oxygen consumption rates equivilant to vehicle treated *ob/ob* levels. While oxygen consumption in iopanoic acid + leptin *ob/ob* mice was restored to control (vehicle *ob/ob* mice) levels, serum thyroid (T_3) levels were not restored to

control levels (vehicle *ob/ob* mice). Furthermore, leptin and iopanoic acid + leptin *ob/ob* mice display elevated radiant body heat relative to both iopanoic acid and vehicle treated *ob/ob* mice. These data suggest that leptin is able to induce thermogenesis and oxygen consumption without upregulating the synthesis of T_3.

Both leptin induced thermogenesis and oxygen consumption rates provide evidence suggestive of metabolic uncoupling, but they do not conclusively demonstrate this phenomena. Stronger evidence of uncoupling protein incorporation into the mitochondrial membrane would include the maintenance of metabolic rate accompanied by a rise in thermogenesis. However since leptin induces enzymes associated with increased metabolism, such as acyl CoA oxidase and carnitine palmitoyl transferase, an increas in metabolism will always accompany a rise in thermogenesis (Wang et al., 1999; Zhou et al., 1997; Zhou et al., 1999). There exists much evidence of leptin's ability to induce metabolic uncoupling. However, with advances in our understanding of leptin's signalling pathways, it is becoming apparent that there exists alternative pathways through which leptin is able to stimulate peripheral tissue metabolic uncoupling. For instance, Mistry et al. (1997)

and Wang et al. (1999) provide support of leptin's ability to stimulate thermogenesis via induction of adrenal synthesis of catecholamines. A rise in catecholamine levels induces lipolytic enzymes and increases fatty acid levels, which in turn induce uncoupling protein synthesis (Weigle et al., 1998). The current study was designed to evaluate a proposed mechanism by which leptin acts on peripheral tissue metabolic expenditure and thermogenesis through the upregulation of thyroid hormone (T_3). The results of the study show that while T_3 levels are suppressed in iopanoic acid treated *ob/ob* mice, leptin is still able to induce both metabolic expenditure and thermogenesis. Further studies should be done to elucidate whether the observed increase in radiant body heat is the result of tissue specific upregulation of uncoupling protein.

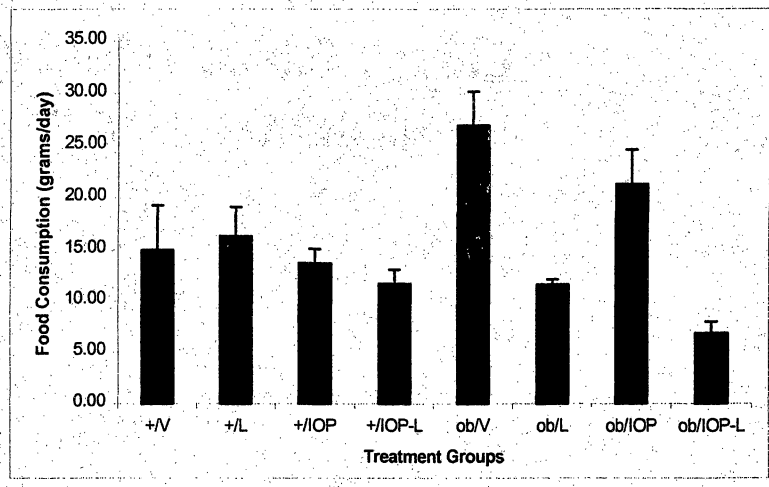
Table 2. Serum thyroid hormone concentrations (T_3 and T_4) and the ratio of T_3/T_4 for day 10 normal (+/?) and obese (*ob/ob*) mice. Vehicle treated obese mice displayed significantly elevated T_3 concentrations with respect to all normal and obese treatment groups. Normal leptin treatment group displayed a reduced serum T_4 concentration with respect to iopanoic acid + leptin treated obese mice. No significant differences were observed in the T_3/T_4 for any treatment groups. An outlier occurred for a single animal in the T_3/T_4 for vehicle treatment (after the division calculation), thus it was removed and a missing data point was generated (n=4 was used to calculate ANOVA). Missing data points occurred in vehicle normal T_3 (n=4 was used to calculate ANOVA), and iopanoic acid obese T_3 (n=4 was used to calculate ANOVA).

		T_3 (ng/dl)	T_4 (µg/dl)	T_3/T_4 (ng/µg)
Normal (+/?)	Vehicle	96.9 ± 21.0	4.0 ± 0.8	23.1 ± 6.5
	Leptin	64.1 ± 33.6	2.4 ± 0.5	28.3 ± 15.4
	Iopanoic Acid	91.8 ± 40.3	3.8 ± 0.8	24.9 ± 9.7
	Iopanoic Acid + Leptin	102.6 ± 9.6	3.2 ± 0.4	32.6 ± 6.52
Obese (ob/ob)	Vehicle	197.1 ± 44.3	3.5 ± 1.7	41.6 ± 8.7
	Leptin	116.8 ± 23.3	3.3 ± 0.5	35.3 ± 6.7
	Iopanoic Acid	100.7 ± 21.2	4.1 ± 1.3	26.2 ± 4.9
	Iopanoic Acid + Leptin	120.7 ± 38.0	4.4 ± 1.2	30.3 ± 17.6

T_3	+/V	+/L	+/IOP	+/IOP-L	ob/L	ob/IOP	ob/IOP-L
ob/V	**S**	**S**	**S**	**S**	**S**	**S**	**S**

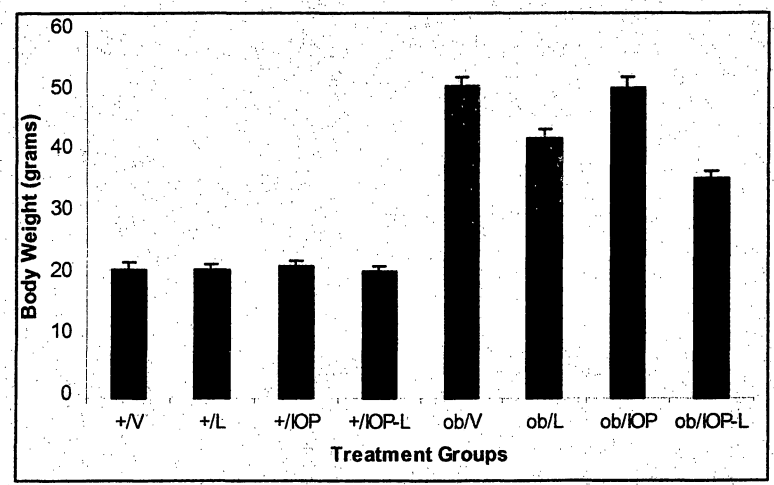
T_4	+/V	+/L	+/IOP	+/IOP-L	ob/L	ob/IOP	ob/IOP-L
ob/IOP-L	**NS**	**S**	**NS**	**NS**	**NS**	**NS**	

Symbols are defined as follows: +/V = vehicle treated +/?; +/L = Leptin treated +/?; +/IOP = Iopanoic acid treated +/?; +/IOP-L = Iopanoic acid plus leptin treated +/?. Obese (*ob/ob*) mice received same treatments but are represented with the symbol ob/__ . Statistical significance is shown in chart below table where treatment groups intersect (S = significance; NS = not significant). T_3 and T_4 statistical significant charts are separate, while no T_3/T_4 chart is shown due to there being no significant difference between treatment groups. All values are mean ± SD.



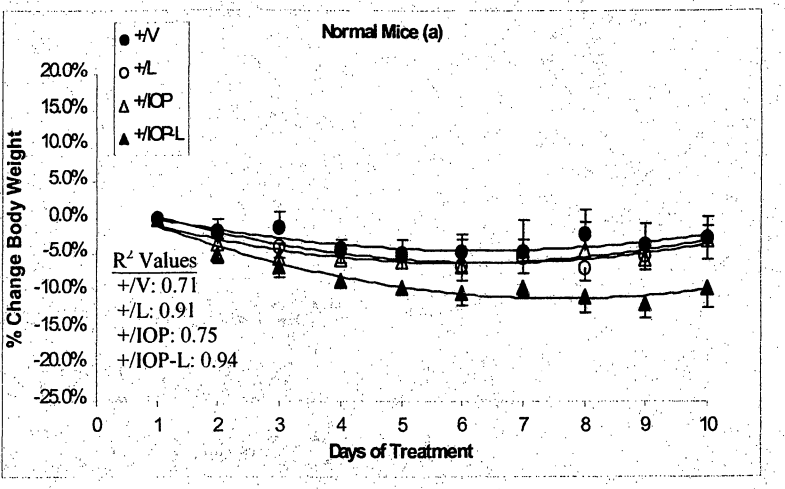
	+/V	+/L	+/IOP	+/IOP-L	ob/L	ob/IOP-L
+/L	NS		NS	NS	NS	S
ob/V	S	S	S	S	S	S
ob/IOP	NS	NS	NS	S	S	S

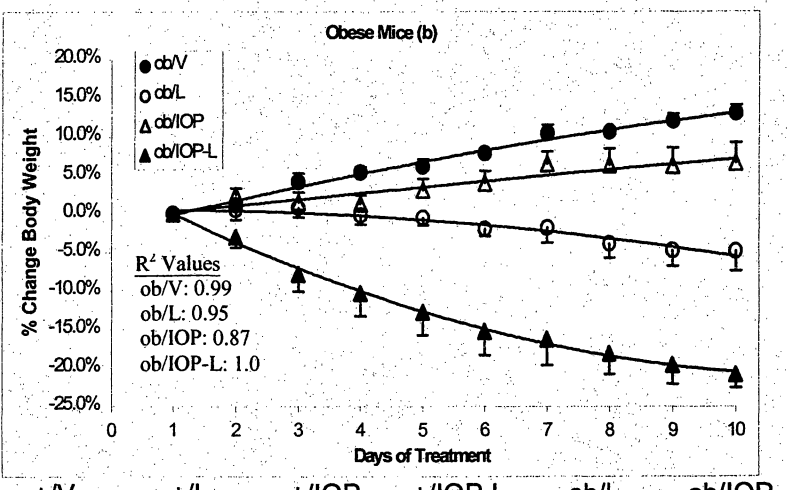
Figure 1. Mean daily food consumption for each treatment group of normal (+/?) and obese (*ob/ob*) mice (grams/day total for each cage). Mean food consumption was analyzed on the last 3 days (days 8-10). Obese (*ob/ob*) mice display increased food consumption as compared to vehicle treated normal (+/?) mice. Leptin treatment in *ob/ob* mice caused a decrease in food consumption relative to +/? vehicle treated mice. Iopanoic acid + leptin treated *ob/ob* mice displayed a decreased food consumption relative to both vehicle treated +/? and iopanoic acid treated *ob/ob* mice. Symbols are defined as follows: +/V= vehicle treated +/?; +/L= Leptin treated +/?; +/IOP= Iopanoic acid treated +/?; +/IOP-L= Iopanoic acid plus leptin treated +/?. Obese (*ob/ob*) mice received the same treatments but are represented with the symbol ob/__. Statistical significance is shown in a chart below the figure where treatment groups intersect (S = significance; NS = not significant). Values are mean $\pm$ SD, n=3.



	+/V	+/L	+/IOP	+/IOP-L	ob/L	ob/IOP-L
ob/V	S	S	S	S	S	S
ob/L	S	S	S	S		S
ob/IOP	S	S	S	S	S	S
ob/IOP-L	S	S	S	S	NS	

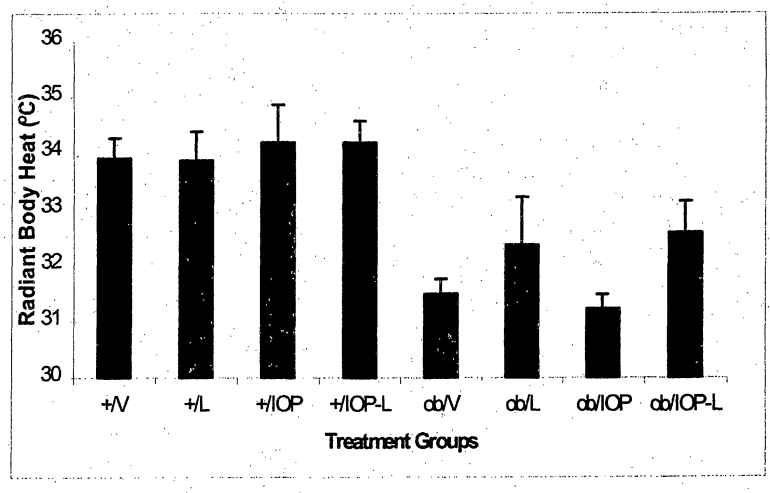
Figure 2. Day 10 body weight for each treatment group of normal (+/?) and obese (*ob/ob*) mice. All normal +/? treatment groups remained the same body weight. Vehicle treated obese mice display body weight higher than vehicle treated normal mice. Leptin treatment in obese mice caused a reduction in body weights with respect to vehicle treated obese mice. Iopanoic acid treated obese mice had higher body weights than both leptin and iopanoic acid + leptin treated obese treatment groups. Iopanoic acid + leptin treated obese mice displayed lower body weights than both leptin and iopanoic acid treatment groups. Symbols are defined as follows: +/V= vehicle treated +/?; +/L= Leptin treated +/?; +/IOP= Iopanoic acid treated +/?; +/IOP-L= Iopanoic acid plus leptin treated +/?. Obese (*ob/ob*) mice received the same treatments but are represented with the symbol ob/__. Statistical significance is shown in a chart below the figure where treatment groups intersect (S = significance; NS = not significant). Values are mean $\pm$ SD, n=5.





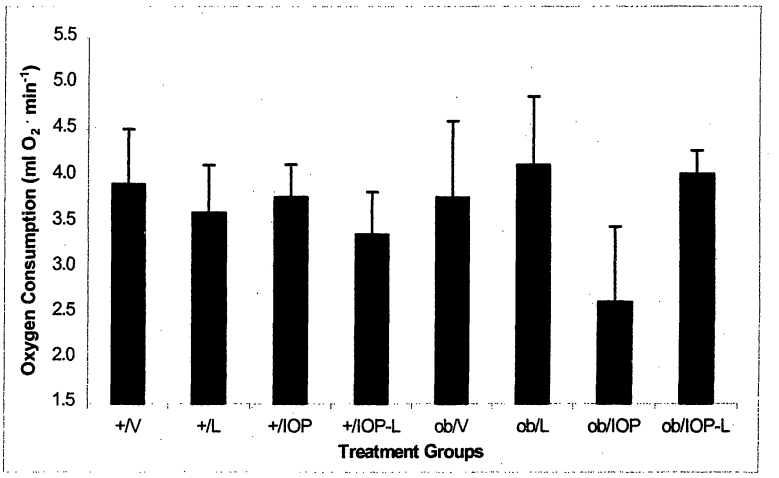
	+/V	+/L	+/IOP	+/IOP-L	ob/L	ob/IOP	ob/IOP-L
+/V		NS	NS	S	NS	S	S
+/L	NS		NS	S	NS	S	S
+/IOP	NS	NS		S	NS	S	S
+/IOP-L	S	S	S		S	S	S
ob/V	S	S	S	S	S	S	S
ob/L	NS	NS	NS	S		S	S
ob/IOP	S	S	S	S	S		S

Figure 3 (a,b). Percent change in body weight with respect to day 1 for normal (a) and obese (b) mice. Mice were treated with either vehicle (V), leptin (L), iopanoic acid (IOP), or iopanoic acid + leptin (IOP-L, lines which correspond to each treatment are displayed in the figures). ANOVA was used to analyze percent change with respect to day 1 of body weights for the final day. In order to statistically evaluate all values of percent change as positive numbers, a constant of 0.25 was added to the percent change of each animal and then arcsin transformation was applied to each the values (Zar,1984). Values are mean $\pm$ standard deviation, n=5.



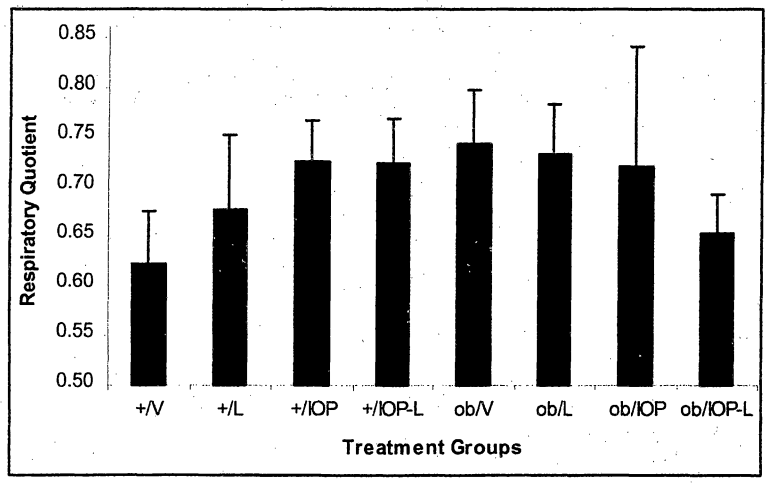
	ob/V	ob/L	ob/IOP	ob/IOP-L
+/V	**S**	**S**	**S**	**S**
+/L	**S**	**S**	**S**	**S**
+/IOP	**S**	**S**	**S**	**S**
+/IOP-L	**S**	**S**	**S**	**S**
ob/L	**NS**		**S**	**NS**
ob/IOP-L	**S**	**NS**	**S**	

Figure 4. Day 10 radiant body heat (°C) for each treatment group of normal (+/?) and obese (*ob/ob*) mice. Normal animals were unchanged by any treatment. Vehicle treated obese mice display a lower radiant body heat than vehicle treated normal mice. Iopanoic acid treated obese mice show a radiant body heat lower than both leptin and iopanoic acid + leptin treated obese mice. Iopanoic acid + leptin treated obese mice have a higher radiant body heat than both vehicle and iopanoic acid treated obese mice. Symbols are defined as follows: +/V= vehicle treated +/?; +/L= Leptin treated +/?; +/IOP= Iopanoic acid treated +/?; +/IOP-L= Iopanoic acid plus leptin treated +/?. Obese (*ob/ob*) mice received the same treatments but are represented with the symbol ob/__. Statistical significance is shown in a chart below the figure where treatment groups intersect
(S = significance; NS = not significant). Values are mean ± SD, n=5.



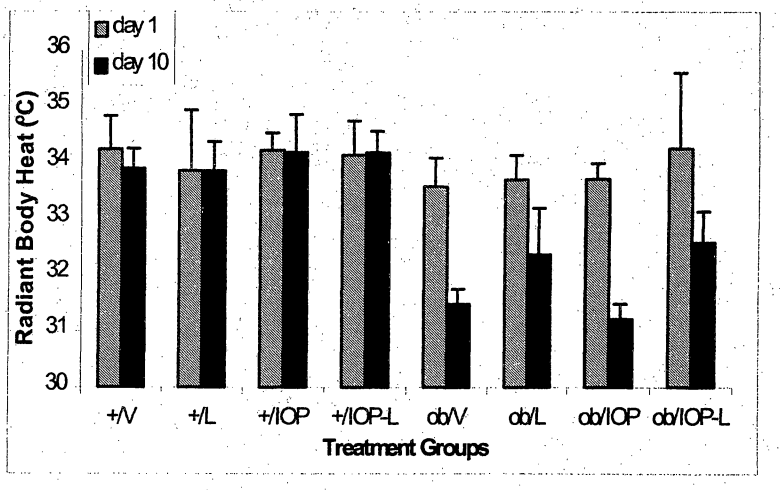
	+/V	+/IOP	ob/V	ob/L	ob/IOP-L
ob/IOP	S	S	S	S	S

Figure 5. Day 10 oxygen consumption rate (ml O_2 . min^{-1}) for day 10 normal (+/?) and obese (*ob/ob*) mice. Iopanoic acid treated obese mice display a reduced oxygen consumption rates relative to the noted treatment groups in the chart. Symbols are defined as follows: +/V (day)= vehicle treated +/?; +/L = Leptin treated +/?; +/IOP = Iopanoic acid treated +/?; +/IOP-L = Iopanoic acid plus leptin treated +/?. Obese (*ob/ob*) mice received the same treatments but are represented with the symbol ob/__. Statistical significance is shown in a chart below the figure where treatment groups intersect (S = significance; NS = not significant). Values are mean ± SD, n=5.



	ob/V	ob/L
+/V	S	S

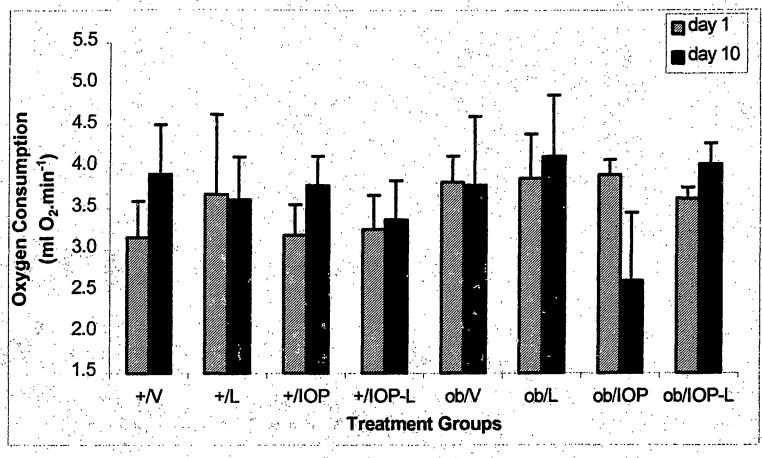
Figure 6. Respiratory quotients (RQ) for day 10 normal (+/?) and obese (*ob/ob*) treatment groups. Day 10 vehicle and leptin treated obese mice display an RQ value above vehicle treated normal mice. Symbols are defined as follows: +/V (day)= vehicle treated +/?; +/L = Leptin treated +/?; +/IOP = Iopanoic acid treated +/?; +/IOP-L = Iopanoic acid plus leptin treated +/?. Obese (*ob/ob*) mice received the same treatments but are represented with the symbol ob/__. Statistical significance is shown in a chart below the table where treatment groups intersect (S = significance; NS = not significant). Values are mean $\pm$ SD, n=5.



	+/V (1)	+/L (1)	+/IOP (1)	+/IOP-L (1)	+/V (10)	+/L (10)	+/IOP (10)
ob/V (10)	S	S	S	S	S	S	S
ob/L (10)	S	S	S	S	S	S	S
ob/IOP (10)	S	S	S	S	S	S	S
ob/IOP-L (10)	S	S	S	S	S	S	S

	+/IOP-L (10)	ob/V (1)	ob/L (1)	ob/IOP (1)	ob/IOP-L (1)	ob/L (10)	ob/IOP-L (10)
ob/V (10)	S	S	S	S	S	NS	S
ob/L (10)	S	S	S	S	S		NS
ob/IOP (10)	S	S	S	S	S	S	S
ob/IOP-L (10)	S	S	S	S	S	NS	

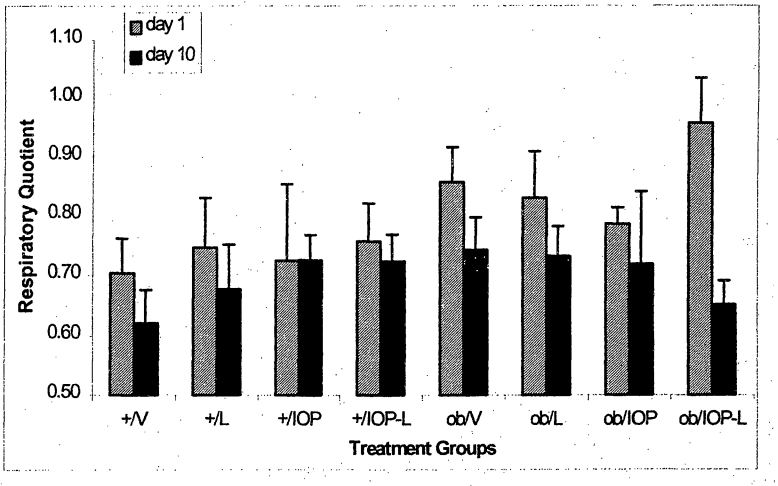
Appendix C. Radiant body heat (°C) for day 1 and day 10 normal (+/?) and obese (*ob/ob*) mice. Radiant body heat is not different between any groups on day 1. There are no significant temperature changes between day 1 and day 10 for any normal treated group. All obese treated groups display decreased radiant body heat from day 1 to day 10. Day 10 iopanoic acid + leptin treated obese mice are significantly higher than day 10 vehicle and day 10 iopanoic acid treated obese mice. Symbols are defined as follows: +/V (day)= vehicle treated +/?; +/L (day)= Leptin treated +/?; +/IOP (day)= Iopanoic acid treated +/?; +/IOP-L (day)= Iopanoic acid plus leptin treated +/?. Obese (*ob/ob*) mice received the same treatments but are represented with the symbol ob/__ (day). Statistical significance is shown in a chart below figure where treatment groups intersect (S = significance; NS = not significant). Statistical analysis was performed using Repeated Measure Analysis of Variance. Values are mean ± SD, n=5.



	+/V (10)	+/L (1)	+/L (10)	+/IOP (10)	ob/V (1)	ob/V (10)
+/V (1)	NS	NS	NS	NS	NS	NS
+/IOP (1)	NS	NS	NS	NS	NS	NS
+/IOP-L (1)	NS	NS	NS	NS	NS	NS
ob/IOP (10)	S	S	S	S	S	S

	ob/L (1)	ob/L (10)	ob/IOP (1)	ob/IOP-L (1)	ob/IOP-L (10)
+/V (1)	NS	S	NS	NS	S
+/IOP (1)	NS	S	NS	NS	NS
+/IOP-L (1)	NS	S	NS	NS	NS
ob/IOP (10)	S	S	S	S	S

Appendix D. Oxygen consumption rates (ml O_2 . min^{-1}) on days 1 and 10 for normal (+/?) and obese mice. All normal and obese mice had equivalant day 1 oxygen consumption rate values. Iopanoic acid treated obese mice display a reduced day 10 oxygen consumption relative to day 1 and all other day 10 obese treatment groups. Symbols are defined as follows: +/V (day)= vehicle treated +/?; +/L (day)= Leptin treated +/?; +/IOP (day)= Iopanoic acid treated +/?; +/IOP-L (day)= Iopanoic acid plus leptin treated +/?. Obese (*ob/ob*) mice received the same treatments but are represented with the symbol ob/__ (day). Statistical significance is shown in a chart below the table where treatment groups intersect (S = significance; NS = not significant). Statistical analysis was performed using Repeated Measure Analysis of Variance. Values are mean ± SD, n=5.



	+/V (1)	+/V (10)	+/L (1)	+/L (10)	+/IOP (1)	+/IOP (10)
ob/V (1)	**NS**	**S**	**NS**	**NS**	**NS**	**NS**
ob/IOP-L (1)	**S**	**S**	**S**	**S**	**S**	**S**

	+/IOP-L (10)	ob/V (10)	ob/L (1)	ob/L (10)	ob/IOP (10)	ob/IOP-L (10)
ob/V (1)	**NS**	**NS**	**NS**	**NS**	**NS**	**S**
ob/IOP-L (1)	**S**	**S**	**S**	**S**	**S**	**S**

Appendix E. Respiratory quotient (RQ) for day 1 and day 10 comparison of normal (+/?) and obese (*ob/ob*) treatment groups. All day 1 treatment groups, except for the iopanoic acid + leptin obes mice, have RQ values that suggest fat metabolism. All day 10 treatment groups have RQ values that indicate fat metabolism. Symbols are defined as follows: +/V (day)= vehicle treated +/?; +/L (day)= Leptin treated +/?; +/IOP (day)= Iopanoic acid treated +/?; +/IOP-L (day)= Iopanoic acid plus leptin treated +/?. Obese (*ob/ob*) mice received same treatments but are represented with the symbol ob/__ (day). Statistical significance is shown in chart below table where treatment groups intersect (S = significance; NS = not significant). Statistical analysis was performed using Repeated Measure Analysis of Variance. Values are mean $\pm$ SD, n=5.

REFERENCES

Auwerx, J., and B. Staels. "Leptin." Lancet 35:737-742, 1998.

Bai, Y., S. Zhang, K. S. Kim, J. K. Lee, and K. H. Kim. "Obese gene expression alters the ability of 30A5 preadipocytes to respond to lipogenic hormones." J. Biol. Chem. 271:13939-13942, 1996.

Baskin, D. G., J. F. Breininger, S. Bonigut, and M. A. Miller. "Leptin binding in the arcuate nucleus is increased during fasting." Brain Research 828:154-158, 1999.

Baskin, D. G., J. F. Breininger, and M. W. Schwartz. Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hpothalamus." Diabetes 48:828-833, 1999.

Boss, O., S. Samec, A. P. Giacobino, C. Rossier, A. Dulloo, J. Seydoux, P. Muzzin, and J. P. Giacobino. "Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression." FEBS Letters. 408:39-42, 1997.

Brand, M. D., L. F. Chien, E. K. Ainscow, D. F. S. Rolfe, and R. K. Porter. "The causes and functions of mitochondrial proton leak." Biochim. Biophys. Acta. 1187:132-139, 1994.

Brown, G. C., P. L. Lakin-Thomas, and M. D. Brand. "Control of respiration and oxidative phosphorylation in isolated rat liver cells." Eur. J. Biochem. 192:355-362, 1990.

Cannon, B., A. Hedin, and J. Nedergaard. "Exclusive occurrence of thermogenin antigen in brown adipose tissue." FEBS Letters 150:129-132, 1982.

Cannon, B., D. Nicholls, and G. L. Lindberg. "Purine nucleotides and fatty acids in energy coupling of mitochondria from brown adipose tissue." Mechanisms in Bioenergetics. Edited by G. F. Azzone, L. Ernster, S. Papa, E. Quagliariello, and N. Siliprand. New York: Academic Press. 1973, pp. 357-363.

Cinti, S., R. C. Frederich, M. C. Zingaretti, R. D. Matteis, J. S. Flier, and B. B. Lowell. "Immunohistochemical localization of leptin and uncoupling protein in white and brown adipose tissue." Endocrinology 138:797-804, 1997.

Cokelaere, M., E. Decuypere, G. Flo, V. M. Darras, and E. R. Kuhn. "Influence of feeding pattern on thyroid hormones in long-term food-restricted rats." Horm. Metab. Res. 28:315-318, 1996.

Cunningham, S., P. Leslie, D. Hopwood, P. Illingworth, R. T. Jung, D. G. Nicholls, N. Peden, J. Rafael, and E. Rial. "The characterization and energetic potential of brown adipose tissue in man." Clinical Science 69:343-348, 1985.

Fehn, R., C. Clark. "Leptin and thyroid hormone in lipid trafficking: a comparison in ob and db mice." FASEB Journal 14:A736, 2000.

Fehn, R., C. Clark, P. Galicia, and B. Underhill. "Leptin restores metabolism by correcting the hypothyroid status of *ob/ob* mice." FASEB Journal 13:A343, 1999.

Fleury, C., M. Neverova, S. Collins, S. Raimbault, O. Champigny, C. Levi-Meyrueis, F. Bouillaud, M. F. Seldin, R. S. Surwit, D. Ricquier, and C. H. Warden. "Uncoupling protein-2: A novel gene linked to obesity and hyperinsulinemia." Nature Genet. 15:269-272, 1997.

Flier, J. S., and B. B. Lowell. "Obesity research springs a proton leak." Genetics 15:223-224, 1997.

Garlid, K. D., D. E. Orosz, M. Modriansky, S. Vassanelli, and P. Jesek. "On the mechanism of fatty acid-induced proton transport by mitochondrial uncoupling protein." J. Biol. Chem. 271:2615-2620, 1996.

Garruti, G., and D. Ricquier. "Analysis of uncoupling protein and its mRNA in adipose tissue deposits of adult humans." Int. J. Obes. Relat. Metab. Disord. 16:383-390, 1993.

Ghibaudi, A. J. J., D. Compton, A. B. Fawzi, C. D. Strader. "Intracerebroventricular injection of leptin increases thermogenesis and mobilizes fat metabolism in *ob/ob*

mice." Horm. Metab. Res. 28:659-663, 1996.

Gimeno, R. E., M. Dembski, X. Weng, N. Deng, A. W. Shyjan, C. J. Gimeno, F. Iris, S. J. Ellis, E. A. Woolf, and L. A. Tartaglia. "Cloning and characterizationof an uncoupling protein homolog: A potential molecular mediator of human thermogenesis." Diabetes 46:900-906, 1997.

Gong, D. W., Y. He, M. Karas, and M. Reitman. "Uncoupling protein-3 is a mediator of thermogenesis regulated by thyroid hormone, beta 3-adrenergic agonists, and leptin." J. Biol. Chem. 272:24129-24132, 1997.

Hadley, M E. "Endocrinology." Prentice-Hall, Inc., 1988 p.325

Hagstrom-Toft, S. Enoksson, E. Moberg, J. Bolinder, P. Arner. "Beta-adrenergic regulation of lipolysis and blood flow in human skeletal muscle *in vivo*." Am. J. Physiol. 275:E909-E916, 1998.

Halaas, J. L., K. S. Gajiwala, M. Maffei, S. L. Cohen, B. T. Chait, D. Rabinowitz, R. L. Lallone, S. K. Burley, J. M. Friedman. "Weight-reducing effects of the plasma protein encoded by the *obese* gene." Science 269:543-546, 1995.

Haynes, G. W., A. D. Morgan, A . S. Walsh, L. A. Mark, I. and W. Sivitz. "Receptor-mediated regional sympathetic nerve activation by leptin." J. Clin. Invest. 100:270-278, 1997.

Heaton, G. M., R. J. Wagenvoord, A. Kemp, and D. G. Nicholls. "Brown adipose tissue mitochondria: photoaffinity labelling of the regulatory site energy dissipation." Eur. J. Biochem. 82:515-521, 1978.

Heim, T., and D. Hull. "The blood flow and oxygen consumption of brown adipose tissue in the new-born rabbit." J. Physiol. London 186:42-55, 1968.

Himms-Hagen, J. "Brown adipose tissue thermogenesis and obesity." Prog. Lipid Res. 28:67-115, 1989.

Himms-Hagen, J. "Brown adipose tissue thermogenesis: Interdisciplinary studies." FASEB Jo. 4:2890-2898, 1990.

Hittelman, K. J., O. Lindberg, and B. Cannon. "Oxidative phosphorylation and compartmentation of fatty acid metabolism in brown fat mitochondria." Eur. J. Biochem. 11:183-192, 1969.

Hua, L., M. Matheny, J. P. Scarpace. "Beta 3-adrenergic-mediated suppression of leptin gene expression in rats." Am. J. Physiol. 273:E1031-E1036, 1997.

Hwang, C. S., and M. D. Lane. "Up-regulation of uncoupling protein-3 by fatty acid in C2C12 myotubes." Biochem. Biophys. Res. Commun. 258:464-469, 1999.

Jezek, P., H. Engstova, M. Zackova, A. E.Vercesi, A. D. T. Costa, P. Arruda, and K. D. Garlid. "Fatty acid cycling mechanism and mitochondrial uncoupling proteins." Biochim. Biophys. Acta. 1365: 319-327, 1998.

Kaplan, M. L., and G. A. Leveille. "Core temperature, O_2 consumption and early detection of *ob/ob* genotype in mice. Am. J. Physiol. 227:912-915, 1974.

Klaus, S., L. Casteilla, F. Bouillaud, D. Ricquier. "The uncoupling protein UCP: A membraneous mitochondrial ion carrier exclusively expressed in brown adipose tissue." Int. J. Biochem. 23:791-801, 1991.

Klingenberg, M. "Dialectics in carrier research: The ADP/ATP carrier and the uncoupling protein." J. Bioenerg. Biomembr. 25:447-457, 1993.

Klingenberg, M. and E. Winkler. "The reconstituted isolated uncoupling protein is a membrane potential driven H^+ translocator." The EMBO Journal 4:3087-3092, 1985.

Kmiec, Z., G. Kotlarz, B. Smiechowska, and A. Mysliwski. "The effect of fasting and refeeding on thyroid follicle structure and thyroid hormone levels in young and old rats." Arc. of Gerentol. and Geriatr. 26:161-175, 1998.

Kotz, C. M., J. E. Briggs, J. D. Pomonis, M. K. Grace, A. S. Levine, and C. J. Billington. "Neural site of leptin influence on neuropeptide Y signalling pathways altering feeding and uncoupling protein. Am. J. Physiol. 275(2pt2):R478-R484, 1998.

Schmidt-Nelson, K. "Animal Physiology." Cambridge University Press, 1990, p. 172, 254.

Lean, M. E., W. P. James, G. Jennings, and P. Trayhurn. "Brown adipose tissue uncoupling protein content in human infants, children and adults." Clin. Sci. (Colch) 71:291-297, 1986.

Lin, C. S., and M. Klingenberg. "Characteristics of the isolated purine nucleotide binding protein from brown fat mitochondria." Biochemistry 21:2950-2960, 1982.

Liu, Q., C. Bai, F. Chen, R. Wang, T. MacDonald, M. Gu, Q. Zhang, M. A. Morsy, and C. T. Caskey. "Uncoupling protein-3: A muscle-specific gene upregulated by leptin in *ob/ob* mice." Gene 207:1-7, 1998.

Lombardi, A., M. Moreno, C. Horst, F. Goglia, and A. Lanni, "Characterization of the binding of 3,3'-di-iodo-L-Thyronine to rat liver mitochondria." J. Endocrin. 154:119-124, 1997.

Mao, W., X. X. Yu, A. Zhong, W. Li, J. Brush, S. W. Sherwood, S. H. Adams, and G. Pan. "UCP4, a novel brain-specific mitochondrial protein that reduces membrane potential in mammalian cells." FEBS Letters. 443:326-330, 1999.

Mason, R., R. Gunst, and J. Hess. "Statistical Design and analysis of experiments." New York, John Wiley & Sons, 1989, p. 301 and 347.

Millet, L., H. Vidal, D. Larrouy, F. Andreelli, M. Laville, and D. Langin, "mRNA expression of the long and short forms of uncoupling protein-3 in obese and lean humans." Diabetologia. 41:829-832, 1998.

Mistry, A. M., A. G. Swick, and D. R. Romsos. "Leptin rapidly lowers food intake and elevates metabolic rates in lean and *ob/ob* mice." J. Nutr. 127:2065-2072, 1997.

Nagase, I., T. Yoshida, K. Kumamoto, T. Umekawa, N. Sakane, H. Nikami, T. Kawada, and M. Saito. "Expression of uncoupling protein in skeletal muscle and white fat of obese mice treated with thermogenic beta 3-adrenergic agonist." J. Clin. Invest. 97:2898-2904, 1996.

Nicholls, D. G., and R. M. Locke. "Thermogenic mechanisms in brown fat." Physiol. Rev. 64:1-64, 1984.

Nobes, C. D., G. C. Brown, P. N. Olive, and M. D. Brand. "Non-ohmic proton conductance of the mitochondrial inner membrane in hepatocytes." J. Biol. Chem. 265:12903-12909, 1990.

Oberkotter, L. V., K. M. Rasmussen. "Changes in plasma thyroid hormone concentrations in chronically food-restricted female rats and their offspring during suckling." J. Nutr. 122:435-441, 1992.

Oh, S. S., M. L. Kaplan. "Early treatment of obese (*ob/ob*) mice with triiodothyronine increases oxygen consumption and temperature and decreases body fat content." Proc. Soc. Exp. Biol. Med. 207:260-267, 1994.

Palmieri, F. "Mitochondrial carrier proteins." FEBS Letters. 346:48-54, 1994.

Pelleymounter, M. A., M. J. Cullen, M.B. Baker, R. W. D. Hecht, B. Thomas, and F. Collins. "Effects of the *obese* gene product on body weight regulation *ob/ob* mice." Science 269:540-543, 1995.

Podolsky, R. J., and F. M. Morales. "The enthalpy change of adenosine triphosphate hydrolysis." J. Biol. Chem. 218: 945-956, 1956.

Prolo, P., M. L. Wong, and J. Licinio. "Leptin." Int. J. Bio. and Cell Bio. 3:1285-1290, 1998.

Prusiner, S., and M. Poe. "Thermodynamic considerations of mammalian thermogenesis." Nature 220:235-237, 1968.

Rafael, J., H. J. Ludolph, and H. J. Hohorst. "Mitochondria from brown adipose tissue: Uncoupling of oxidative phosphorylation by long chain fatty acids and

recoupling by guanosine triphosphate." Hoppe-Seyler's Z. Physiol. Chem. 350:1121-1131, 1969.

Rolfe, D. F. S., G. C. Brown. "Cellular energy utilization and molecular origin of standard metabolic rate in mammals." Physiol. Rev. 77:731-758, 1997.

Rolfe, D. F. S., and M. D. Brand. "The contribution of mitochondrial proton leak to the rat of respiration in rat skeletal muscle and to SMR." Am. J. Physiol. 271:C1380-C1389, 1996.

Rolfe, D. F. S., A. J. Hulbert, and M. D. Brand. "Characteristics of mitochondrial proton leak and control of oxidative phosphorylation in the major oxygen consuming tissues of the rat. Biochim. Biophys. Acta. 1188:405-416, 1994.

Satoh, N., O. Yoshihiro, G. Katsuura, Y. Numata, T. Tsuji, M. Hayase, K. Ebihara, H. Masazaki, K. Hosoda, Y. Yoshimasa, and K. Nakao. "Sympathetic activation of leptin via the ventromedial hypothalamus." Diabetes 48:1787-1793, 1999.

Schmidt-Neilsen, Knut. "Animal Physiology: Adaption and Environment." Press Syndicate of the University of Cambridge, 1990, p. 172.

Shimabukuro, M., K. Koyama, G. Chen, M. Y. Wang, F. Trieu, Y. Lee, C. B. Newgard, and R. H. Unger. "Direct antidiabetic effect of leptin through triglyceride depletion of tissues." Proc. Nat. Acad. Sci. USA. 94:4637-4641, 1997.

Solanes, G., A. Vidal-Puig, D. Grujic, J. S. Flier, and B. B. Lowell. "The human uncoupling protein-3 gene. Genomic structure, chromosomal localization, and genetic basis for short and long form transcripts." J. Biol. Chem. 272:25433-25436, 1997.

Vidal-Puig, A., G. Solanes, D. Grujic, J. S. Flier, and B. B. Lowell. "UCP3: An uncoupling protein homologue expressed preferentially and abundantly in skeletal muscle and brown adipose tissue." Biochem. Biophys. Res. Commun. 235:79-82, 1997.

Wang, M.-Y., Y. Lee, and R. H. Unger. "Novel form of lipolysis induced by leptin." J. of Bio. Chem. 274:17541-17544, 1999.

Weigle, D. S., L. E. Selfridge, M. W. Schwartz, R. J. Weely, D. E. Cummings, P. J. Havel, J. L. Kuijper, and H. Beltrandelrio. "Elevated free fatty acids induce uncoupling protein 3 expression in muscle." Diabetes 47:298-302, 1998.

Zar, J. H. "Biostatistical Analysis." Prentice-Hall, Inc. 1984. p. 216, 238.

Zhou, Y. T., M. Shimabukuro, K. Koyama, Y. Lee, M. Y. Wang, F. Trieu, C. B. Newgard, and R. H. Unger. "Induction by leptin of uncoupling protein-2 and enzymes of fatty acid oxidation." Proc. Nat. Acad. Sci. USA. 94:6386-6390, 1997.

Zhou, Y. T., M. Shimabukuro, K. Koyama, Y. Lee, M. Y. Wang, F. Trieu, C. B. Newgard, and R. H. Unger. "Reversing adipocyte differentiation: Implications for treatment of obesity." Proc Natl. Acad. Sci. USA. 96:2391-2395, 1999.